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Effects of Cytochrome P450-Interacting Plant Growth Retardants, Fungicides and Related Compounds on Cell Development and Phase-I Biotransformation Capacity of Unicellular Photoautotrophic Green Algae

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Abstract: Fourteen compounds (paclobutrazol, triadimenol, BAS111..W, propiconazole, tetcyclacis, prochloraz, metyrapone, piperonyl butoxide, 1-aminobenzotriazole, fenpropimorph, propham, prohexadione, mepiquat chloride and chlormequat chloride), most of them established inhibitors of cytochrome P450-dependent mixed function oxygenases and used as pesticides, especially plant growth regulators or fungicides, were applied to the non-target organisms *Chlorella fusca* and *Chlorella sorokiniana*, two species of photoautotrophic unicellular green algae. The inhibitory properties of these compounds were evaluated by comparing concentration/response relationships for the integral parameters of cell volume growth and cell division with those for the P450-dependent *O*-dealkylase activity measured *in vivo* using 7-ethoxycoumarin and 7-ethoxyresorufin as xenobiotic model substrates for phase-I biotransformation. The results obtained indicate a strong algicidal activity for some of these compounds, with differential sensitivity of the order: cell division > *O*-dealkylation > cell volume increase. EC_{50} values for cell division of *C. fusca* ranged from 0·1 to 9·3 μmol litre^{-1} for prochloraz and paclobutrazol, respectively. Furthermore, in most cases, concentrations around 10 μmol litre^{-1} limited significantly the capacity for cytochrome P450 *O*-dealkylase activity.

Key words: algae, non-target toxicity, cytochrome P450, ethoxycoumarin-*O*-dealkylase, ethoxyresorufin-*O*-dealkylase, plant growth retardants, fungicides

1 INTRODUCTION

Cytochrome P450-dependent enzymes (P450s) of plants are known to be involved in both the biosynthesis of essential secondary metabolites and the (de)toxification of pesticides and other xenobiotic compounds.[1] In the latter case, these transformations are termed 'phase-I' reactions.[2] It is widely accepted that distinct isoforms of plant P450s exist which participate in the metabolic transformation of certain physiological substrates which include phenylpropanoids, fatty acids, cyanogenic glycosides and terpenes. Terpene metabolism is especially significant because derivatives such as gibberellins (GAs) and sterols act as hormones or are essential plant constituents, respectively. GAs and sterols are involved in the regulation of shoot histogenesis of higher plants by triggering cell elongation and cell division.[3] These cytochrome P450s are target sites for the control of plant growth and development by specific effectors, by plant growth regulators and retardants. Structurally related fungicides act by inhibiting specifically the P450-mediated 14α-demethylation step in the formation of sterols in fungi.

The important role of cytochrome P450-related enzymes in the biotransformation of xenobiotics in plants is now also well established. Plant P450-

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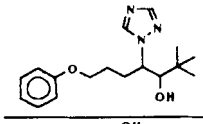
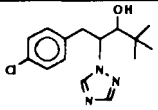
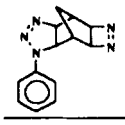
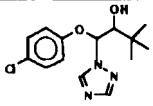
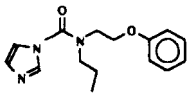
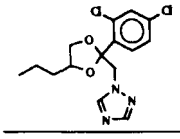
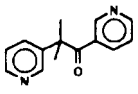
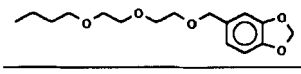
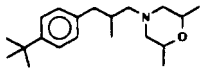
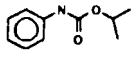
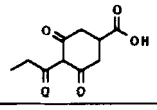
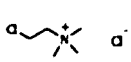
TABLE 1

Substances Tested as Potential Inhibitors of Cell Growth, Cell Reproduction, ECOD- and EROD-Activity of *Chlorella fusca* and *Chlorella sorokiniana*.

Structure	*Common name*	*(Use)*[a]	*Mode of action*	*[Ref.]*[b]
	BAS 111.W	(r*)	Gibberellin and sterol biosynthesis inhibitor (inhibits Cyt P450 mediated *ent*-kaurene oxidation and 14α-demethylation)	**[8, 9]**
	Paclobutrazol	(r)	Gibberellin and sterol biosynthesis inhibitor (inhibits Cyt P450 mediated *ent*-kaurene oxidation and 14α-demethylation)	**[15, 16]**
	Tetcyclacis	(r)	Gibberellin and sterol biosynthesis inhibitor (inhibits Cyt P450 mediated *ent*-kaurene oxidation and 14α-demethylation)	**[8, 17]**
	Triadimenol	(f)	Gibberellin and sterol biosynthesis inhibitor (inhibits Cyt P450 mediated *ent*-kaurene oxidation and 14α-demethylation)	**[15, 18]**
	Prochloraz	(f)	Sterol biosynthesis inhibitor (inhibits Cyt P450 mediated 14α-demethylation)	[19]
	Propiconazole	(r)	Sterol biosynthesis inhibitor (inhibits Cyt P450 mediated 14α-demethylation)	**[9, 20]**
	Metyrapone	( )	Sterol biosynthesis inhibitor (inhibits Cyt P450 mediated ecdysone 20-monooxygenase)	[21]
	Piperonyl butoxide	(is)	Cyt P450 inhibitor (forms a stable inactive complex with cytochrome P450)	[11]
	1-Aminobenzotriazole	( )	Suicide substrate of Cyt P450 (inhibits cinnamic acid 4-hydroxylase)	[10, **22**]
	Fenpropimorph	(f)	Sterol biosynthesis inhibitor (inhibits cycloeucalenol-obtusifoliol isomerase, $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase)	**[23]**
	Propham	(h)	Mitosis inhibitor (interferes with the mitotic spindle)	[24]
	Prohexadione	(r)	Gibberellin biosynthesis inhibitor (inhibits 2-oxoglutarate-depending dioxygenases)	**[25]**
	Mepiquat chloride	(r)	Gibberellin biosynthesis inhibitor (inhibits *ent*-kaurene synthetases A, B)	**[26]**
	Chlormequat chloride	(r)	Gibberellin biosynthesis inhibitor (inhibits *ent*-kaurene synthetases A, B)	**[26]**

[a] * Reference 8; others, Reference 27. f = fungicide, h = herbicide, is = insecticide synergist, r = plant growth regulator. [b] Bold typed references document evidence for interactions with plant secondary metabolism.

mediated hydroxylations, epoxidations and *N*- and *O*-dealkylations of exogenous compounds have been documented.[4–7]

Inhibition of cytochrome P450s by plant growth regulators containing nitrogen heterocycles has been attributed to a strong interaction of the cytochrome's protoheme iron with lone pairs of electrons of the sp^2-hybridized nitrogen.[8,9] Mechanism-based cytochrome P450 inhibitors, such as 1-aminobenzotriazole (ABT) or piperonyl butoxide, first require activation to form the active cytochrome P450-interfering species.[10,11] These modes of inhibition suggest that it is not possible to differentiate precisely between the P450 isoforms involved in biosyntheses and in the biotransformation of xenobiotics; in particular, the latter should not explicitly be targeted. However, in plant cell suspension cultures, tetcyclacis, paclobutrazol and ABT have all been shown to inhibit strongly the P450-mediated *N*-demethylation of the urea-type herbicide chlorotoluron, but the selectivity of such inhibitors for different P450-catalysed reactions is not entirely clear.[12,13]

In the present paper concentration/response data of 14 compounds (Table 1) for two green algae, *Chlorella fusca* and *Chlorella sorokiniana*, are shown discriminating between cell volume growth and cell division as integral physiological parameters. These data are compared to effects on the apparent P450-dependent *O*-dealkylase activities of both algal species measured *in vivo* by adapting a common method for the determination of P450-dependent phase-I biotransformation activities, specifically using 7-ethoxycoumarin and 7-ethoxyresorufin as xenobiotic model substrates.[14] The results are discussed in terms of (a) the algicidal properties of the chosen compounds, most of which are used as plant growth regulators and fungicides, and (b) the further inhibitory activities of these compounds on the cytochrome P450-dependent phase-I biotransformation capacity.

2 MATERIAL AND METHODS

2.1 Chemicals

7-Ethoxyresorufin (7-ER) was synthesized from resorufin as described by Klotz *et al.*[28] The sodium salt of resorufin was purchased from Sigma (Deisenhofen, Germany), and 1-iodoethane was from Merck (Darmstadt, Germany). Preparative separation was performed on a 310-25 LiChroprep Si60 column (Merck, Darmstadt, Germany). 7-ER was judged better than 98% pure by HPLC using a combination of diode array and fluorescence detection. The analytical standard was obtained from Boehringer (Mannheim, Germany). Stock solutions of 7-ER in acetone were stored at −30°C. Sources of other chemicals were as follows: 1-aminobenzotriazole (Aldrich, Steinheim, Germany); paclobutrazol (Promochem, Wesel, Germany); 7-ethoxycoumarin (7-EC), umbelliferone, metyrapone, propham, cholesterol, polyoxyethanyl-cholesteryl sebacate (30% cholesterol) and ergosterol (Sigma, Deisenhofen, Germany); BAS 111..W, prohexadione calcium and tetcyclacis (BASF, Limburgerhof, Germany); all other pesticides and organic solvents (analytical reagent or HPLC grade) from Riedel-deHaen (Seelze/Hannover, Germany). Ingredients required for algal nutrient media were obtained from Merck.

2.2 Organisms and culture conditions

The unicellular green alga *Chlorella fusca* var. *vacuolata* Shih. et Krauss, strain 211-15, culture collection Pringsheim (Göttingen, Germany) was grown photoautotrophically at 28(±0·5)°C in an appropriate sterilized medium adjusted to pH 7·6 (10 mM Na phosphate buffer).[29] Cultures were aerated with sterilized, water-saturated air, enriched with carbon dioxide (1·5–2·0%, v/v) and illuminated by a combination of two types of fluorescent tube lights (L36W/41 Interna, L36W/11 daylight; Osram, Berlin, Germany) with an intensity of 13–18 W m^{-2} (22–33 klux). Cells were synchronized by light : dark changes of 14 : 10 h and a periodic dilution to a standard cell density of 1×10^6 ml^{-1}.

Chlorella sorokiniana, strain 211-8k, culture collection Pringsheim (Göttingen, Germany) was cultivated at 37(±0·5)°C synchronized by light : dark changes of 10 : 14 h and a periodic dilution to a standard cell density of 1×10^6 ml^{-1}. All other conditions were the same as for *C. fusca*.

Batch cultures of both algal species were cultivated corresponding to synchronized conditions but illuminated for 24 h and diluted daily to a standard cell density of 5×10^6 ml^{-1}.

Cell number and cell volume distribution were analysed using a Coulter Counter Model ZB Industrial and a Coulter Channelizer C-256 (Coulter Electronic, Ltd., Luton, Beds., UK). For statistical treatment (mean cell volume of population, calculated as median or average of cell volume distribution) data were transferred directly to a microcomputer.

2.3 Determination of concentration/response relationships

Concentration/response relationships of the test compounds were determined in a 24-h bioassay under synchronized conditions using cell volume growth (at t_{14} for *C. fusca* and t_{10} for *C. sorokiniana*) and cell division (t_{24}) as effect parameters.[30,31] Effective concentrations and confidence limits were statistically determined by a two-parameter Weibull model.[32]

2.4 *O*-Dealkylase assay

Acetone stock solution of 7-ethoxyresorufin (7-ER) or 7-ethoxycoumarin (7-EC) was transferred into an Erlenmeyer flask and the acetone removed by evaporation prior to filling the flask completely with double-distilled water, resulting in a concentration of 20 μM for 7-ER and 200 μM for 7-EC. The flask was then closed and incubated for about 3 h at 50°C in the dark with continuous stirring until the ether was redissolved. Solutions of pesticides were prepared in the same way to a concentration of 200 μM. To each of 15 ml 7-ER (7-EC) solution a volume of the pesticide solution and/or double-distilled water was added sufficient to obtain the solution (30 ml) of substrate/inhibitor needed for the *O*-dealkylase assay.

Batch-cultivated algae were harvested (3250*g*, 5 min), washed twice, resuspended in nutrient medium and adjusted to a biovolume of 1·0($\pm$0·02) μl ml^{-1} (average cell volume × cell number). Aliquots (2·5 ml) of algal suspension were placed in 10 ml centrifuge tubes containing a 15 mm stirrer bar. Test-tubes were placed in a waterbath at 28°C with a multipoint magnetic stirrer (Variomag : H + P, Munich, Germany) adjusted to maximum speed and illuminated as described above. The design made it possible to test simultaneously up to 48 samples. The assay was started by adding 2·5 ml substrate/inhibitor solution to each test-tube, resulting in a final biovolume of 0·5($\pm$0·01) μl ml^{-1}, a final concentration of the *O*-dealkylase substrate of 5·0 μM 7-ER, 50 μM 7-EC and final concentrations (0–50 μM) of the inhibitor tested. After an incubation period of 60 min (10 min in the case of ethoxyresorufin-*O*-dealkylase, EROD, in *C. sorokiniana*[14]) the algae were pelleted in a precooled (0°C) centrifuge at 3250*g* for 5 min. Supernatants were analysed by HPLC as described previously, slightly modified by using a shorter analytical column [Aluspher 100 RP select B (75 mm × 4 mm ID), Merck] which allowed running times $\leqq$ 1 min.[33]

2.5 Determination of photosynthetic O_2 production

Photosynthetic O_2 production was determined polarographically using a jacketed glassware cell equipped with a Clark-type electrode (Gilson, Middleton, WI, USA) connected to a model 5300 biological oxygen monitor (YSI, Yellow Springs, Ohio, USA) with microcomputer-aided data processing.

3 RESULTS

3.1 Algicidal properties of test compounds

Table 2 lists data comparing between inhibition of cell growth and cell division by the 14 test compounds. These data were derived from concentration/response curves of *C. fusca* and *C. sorokiniana* with the various compounds shown in Table 1. Concentrations giving 50% inhibition of cell volume growth ranged from 5·0 μmol litre^{-1} to 0·5 mmol litre^{-1}. Prochloraz and, especially, tetcyclacis had a strong inhibitory action on algal cell growth, whereas the triazole derivatives and prohexadione were the least algitoxic compounds in this series, apart from the two additionally tested onium compounds, mepiquat and chlormequat, which showed no effect on cell development. 1-Aminobenzotriazole at 2·0 mmol litre^{-1} caused only slight inhibition (15–20%) of cell volume growth of both algal strains. Significant differences in sensitivity were seen between the two species for some compounds. *C. fusca* was 10-fold more sensitive to piperonyl butoxide and by contrast *C. sorokiniana* was about 6-fold more sensitive to fenpropimorph.

The corresponding data for the inhibition of cell division at t_{24} show different rankings and sensitivities. The triazole derivatives, tetcyclacis and fenpropimorph caused 50% inhibition of cell division of *C. fusca* at micromolar concentrations, and prochloraz was particularly potent, with an EC_{50} of 0·1 μmol litre^{-1}. The corresponding EC_{90}/EC_{10} ratio describes the slope of the original concentration/response curves. In most of the cases complete inhibition of cell division of *C. fusca* was obtained at micromolar concentrations of these compounds.

This distinct difference in the sensitivities of cell volume growth and cell division is manifested in the ratio of the corresponding EC_{50} values: a significant independent inhibition of cell division will be reflected by a ratio of $\geqq$ 5·0. The corresponding ratios determined for propham are unexpectedly small, given the proposed mode of action as a mitosis inhibitor.

In contrast, the data show that *C. sorokiniana* is more resistant, as seen in the comparatively higher concentrations needed for the inhibition of cell division.

Figure 1 illustrates the results of experiments performed by delaying the time of application of selected compounds within the individual cell cycles of both algal species. When applied at t_0, the test concentrations caused complete inhibition of cell division at t_{24}. Inhibition of *C. fusca* remained unchanged for applications performed up to t_{10}. Applications later than t_{10} gave decreasing inhibition. No effect was observed when adding the compounds at the end of the illumination period.

By contrast, for complete inhibition of cell division in *C. sorokiniana*, the tested compounds had to be applied before t_2. For prochloraz and propiconazole, the application delayed by only two hours (at t_4) diminished the inhibitory effects. Among the compounds studied, triadimenol was still affecting cell division when added at t_{10} to t_{12}. The morpholine-type fungicide, fenpropimorph, however, completely inhibited cell division of *C.*

TABLE 2
Effect Concentrations of Different Retardants, Fungicides and Related Compounds for 50% Inhibition of Cell Volume Growth and Cell Division of *Chlorella fusca* and *Chlorella sorokiniana*.

		Inhibition of Cell volume growth			*Inhibition of cell division*			
Compound	*Alga*	EC_{50}[a] (*μmol litre*$^{-1}$)		$\frac{EC_{90}}{EC_{10}}$	EC_{50}[a] (*μmol litre*$^{-1}$)		$\frac{EC_{90}}{EC_{10}}$	$\frac{EC_{50}\ growth}{EC_{50}\ division}$
BAS 111..W	*C. fusca*	474	(311–569)	5·0	3·2	(1·6–5·3)	34·9	148·0
	C. soro.	506	(485–530)	1·4	250	(100–480)	4·8	2·0
Paclobutrazol	*C. fusca*	180	(160–207)	11·5	9·3	(9·0–9·7)	6·1	19·4
	C. soro.	238	(202–324)	nd	20	(17–23)	11·0	11·9
Triadimenol	*C. fusca*	416	(383–452)	7·9	6	(5·1–6·9)	3·4	69·3
	C. soro.	252	(141–373)	4·4	50	(19–82)	7·0	5·0
Propiconazole	*C. fusca*	81	(78–85)	2·5	0·5	(0·5–0·6)	3·9	162·0
	C. soro.	101	(93–110)	3·1	1·2	(1·0–1·3)	8·0	84·2
Tetcyclacis	*C. fusca*	5·2	(3·3–18)	45·5	0·9	(0·8–1·2)	1·7	5·8
	C. soro.	6·3	(4·4–10)	19·6	4·2	(1·0–26)	55·6	1·5
Prochloraz	*C. fusca*	36	(32–41)	10·0	0·1	(0·1–0·1)	2·0	360·0
	C. soro.	18	(13–24)	16·9	1·6	(1·3–2·3)	7·3	11·3
1-Aminobenzotriazole	*C. fusca*	>2000[b]	—	—	>2000[b]	—	—	—
	C. soro.	>2000[b]	—	—	>2000[b]	—	—	—
Metyrapone	*C. fusca*	266	(186–398)	8·4	48	(47–49)	1·2	5·5
	C. soro.	538	(403–838)	13·9	478	(376–632)	11·8	1·1
Piperonyl butoxide	*C. fusca*	44	(35–53)	11·9	3·4	(2·4–7·6)	4·2	12·9
	C. soro.	393	(nd)[c]	nd	68	(28–212)	36·1	5·8
Mepiquat	*C. fusca*	>5000[b]	—	—	>5000[b]	—	—	—
	C. soro.	>5000[b]	—	—	>5000[b]	—	—	—
Chlormequat	*C. fusca*	>5000[b]	—	—	>5000[b]	—	—	—
	C. soro.	>5000[b]	—	—	>5000[b]	—	—	—
Prohexadione	*C. fusca*	296	(279–313)	5·3	152	(151–153)	4·2	1·9
	C. soro.	210	(200–220)	1·2	195	(184–207)	3·0	1·1
Fenpropimorph	*C. fusca*	60	(nd)[c]	nd	0·5	(0·3–0·5)	5·0	120·0
	C. soro.	10	(9·3–13)	4·8	0·1	(0·05–0·2)	1·2	100·0
Propham	*C. fusca*	111	(100–123)	21·1	43	(38–47)	7·9	2·6
	C. soro.	84	(78–90)	22·9	16	(15–17)	20·5	5·3

Concentration/response data were determined in a 24-h bioassay under synchronized conditions (stated in the text) and statistically treated using a two-parameter Weibull model.
[a] 95% confidence limits given in parenthesis.
[b] Highest concentration tested.
[c] nd: not determined (insufficient solubility).

sorokiniana when applied as late as t_8; cell division was still affected when this compound was added after the light : dark change, during t_{10} to t_{12}. At this time the proliferation of autospores had already started and was completed approximately two hours later. It is important to note that the test concentrations applied in these trials caused no inhibition of cell volume growth.

Attempts to overcome the inhibition of cell division caused by triadimenol, prochloraz, propiconazole, fenpropimorph and prohexadione by adding gibberellic acid (GA_3), ergosterol or cholesterol (each at 0·1 mM) were not successful. With respect to the weak acid behaviour of GA_3 these trials were made under a pH regime of 5·2, which was tolerated by both algal strains.

3.2 Inhibition of algal EROD and ECOD activities *in vivo*

The *in-vivo* *O*-dealkylase activities of untreated batch cultures of *C. fusca* and *C. sorokiniana* were estimated by determining the appearance of the metabolites—resorufin in the case of 7-ethoxyresorufin-*O*-dealkylase (EROD) and umbelliferone in the case of 7-ethoxycoumarin-*O*-dealkylase (ECOD)—in the growth media. Apparent activities are summarized as follows: ECOD of *C. fusca* 0·64(±0·16) nmol min^{-1}, EROD of *C. fusca* 1·3(±0·33) nmol min^{-1}, ECOD of *C. sorokiniana* 0·62(±0·2) nmol min^{-1}, EROD of *C. sorokiniana* 10·1(±3·2) nmol min^{-1}. The relatively high values of the corresponding standard deviations are linked to the

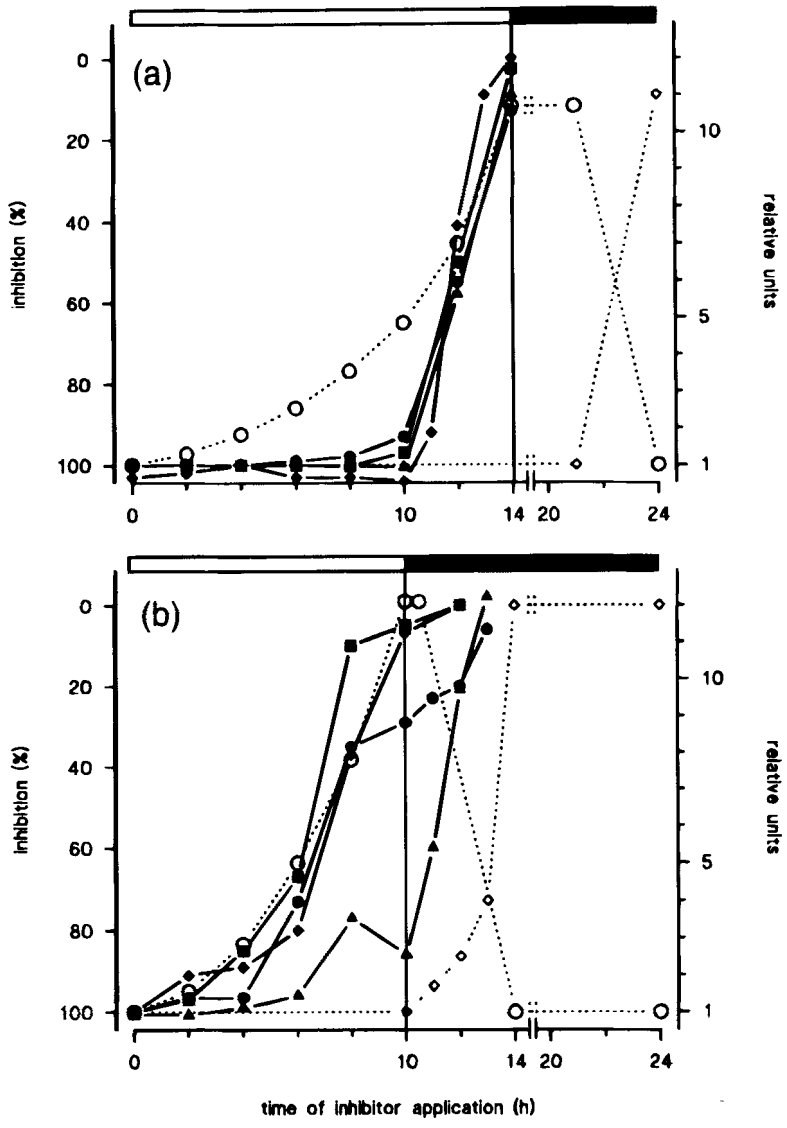


Fig. 1. Delayed times of application of selected compounds and the inhibitory effects on cell division at t_{24} of (a) *Chlorella fusca* and (b) *Chlorella sorokiniana*. Open and solid bars at the top indicate the periods of illumination and darkness, respectively. The open symbols reflect the typical stages of cell development (relative units) within the individual cell cycles under synchronized conditions (see text). (○) median of cell volume distribution, (◇) cell number. *C. fusca:* (●) triadimenol (2×10^{-5} M), (■) propiconazole (5×10^{-6} M), (◆) prochloraz (1×10^{-6} M), (▲) fenpropimorph (5×10^{-6} M) *C. soro.:* (●) triadimenol (1×10^{-4} M), (■) propiconazole (5×10^{-6} M), (◆) prochloraz (3×10^{-6} M), (▲) fenpropimorph (1×10^{-6} M).

batch variation, permanent illumination leading to a high variability in the cell volume distribution which is related to the *O*-dealkylase activities.[14] The intra-batch variations of the *O*-dealkylase assays are much smaller; here the relative standard deviations are commonly less than 5%.

Figure 2 presents the changes of algal ethoxyresorufin- and ethoxycoumarin-*O*-deethylase activities *in vivo* caused by increasing concentrations of the test compounds. All substances known to be cytochrome P450 inhibitors (see Table 1) inhibited significantly the *O*-dealkylation of both the resorufin and coumarin ethers, although there were distinct differences in the sensitivities between the two algal strains and between EROD and ECOD activity, respectively. In most cases ECOD of *C. sorokiniana* was more sensitive to these compounds than ECOD of *C. fusca*. In *C. fusca*, EROD tended to be more sensitive than ECOD whereas for *C. sorokiniana*, responses to EROD and ECOD were similar.

In most cases the highest inhibitor concentration tested (50 μM) on *O*-dealkylase-activity caused little or no effect on cell volume growth determined under synchronized conditions (Table 2). With prochloraz, fenpropimorph and tetcyclacis, however, secondary effects on the P450-mediated *O*-dealkylases cannot be excluded. Determinations of algal photosynthetic oxygen production with inhibitors present (50 μM, 1 h incubated under the conditions of the *O*-dealkylase-assay) showed no effect compared to untreated controls (data not shown). Therefore NADP(H) essentially needed as co-substrate for dealkylation should not be

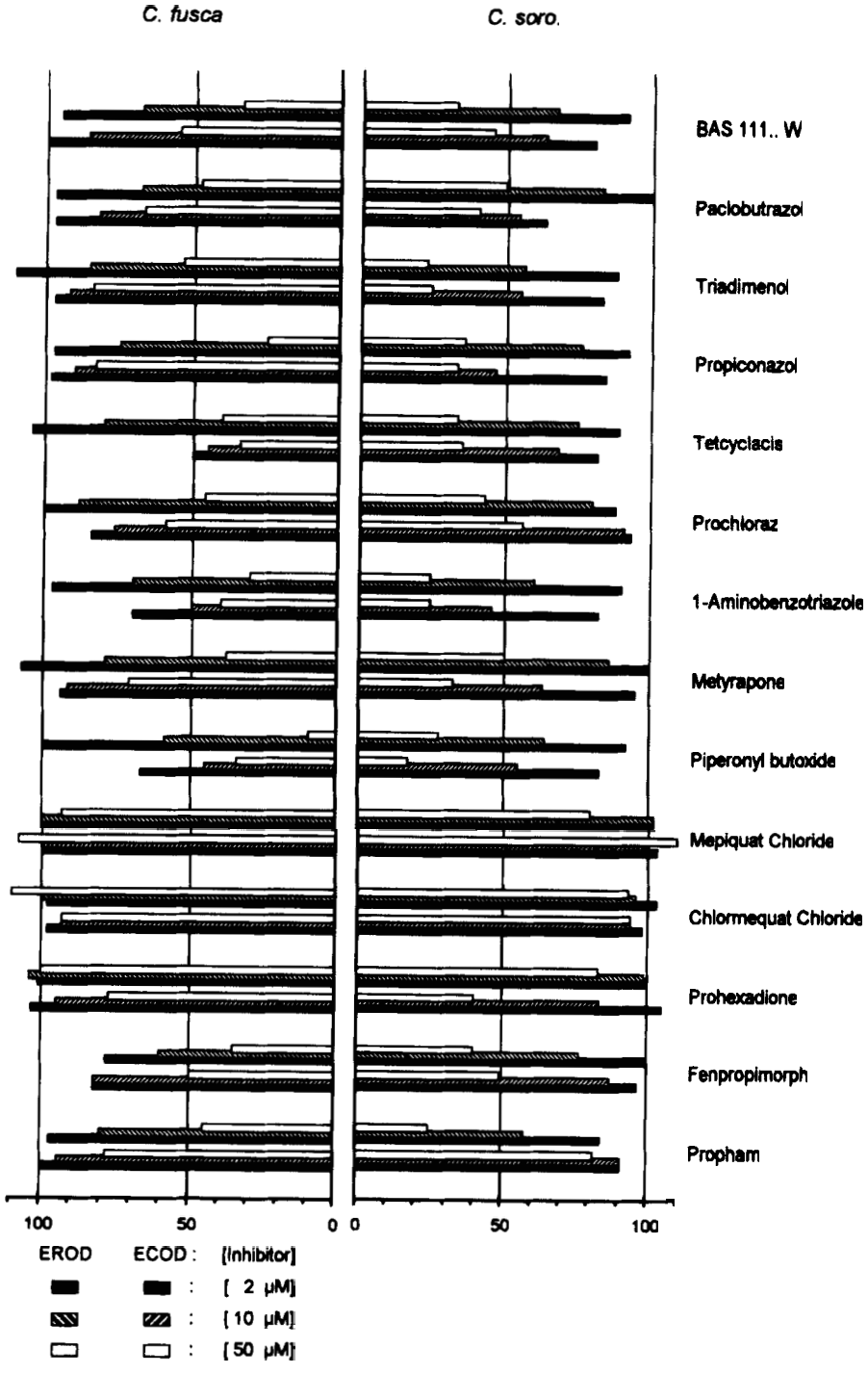


Fig. 2. Relative apparent activities of 7-ethoxyresorufin-*O*-dealkylase (EROD) and 7-ethoxycoumarin-*O*-dealkylase (ECOD) of *Chlorella fusca* and *Chlorella sorokiniana* after the application of the test compounds. Values are means of four determinations. Conditions of the *in-vivo O*-dealkylase assays are as stated in the text.

limited by any of the test compounds.

Of the other compounds, not known to be cytochrome P450 inhibitors, which included the onium-type and acylcyclohexanedione-type gibberellin biosynthesis inhibitors, only prohexadione caused *O*-dealkylase inhibition, significant for EROD of both species. Fenpropimorph and propham, although not documented as P450 inhibitors, evidently inhibited algal *O*-dealkylases, while propham discriminated between EROD and ECOD.

4 DISCUSSION

Most of the compounds tested in the present series, excepting 1-aminobenzotriazole and the onium-type growth retardants mepiquat chloride and chlormequat chloride, showed strong toxic properties against algae.

In most cases the distinct differences in the sensitivities of cell volume growth and cell division as integral physiological parameters indicate a specific interaction of the test compounds with physiological processes related to and essential for cell division. Similar phytotoxic effects on higher plants caused by these agents are more specifically related to changes in the content and composition of sterols and/or derivatives of gibberellin (Table 1).[34] An alteration of the sterol content caused by sterol biosynthesis inhibitors possibly affects membrane fluidity which might be specifically related to the process of cell division.[2] Whereas the presence of GAs in algae is not known so far, the existence and composition of sterols and sterol derivatives in *Chlorella ssp.* is now well documented.[35,36] However, the sterols detected in algae differ qualitatively from those of higher plants. Fungisterol and schotenol are the main membrane constituents in *Chlorella kessleri*.[37] Thus the inability to reverse the inhibition of cell division by a simple substitution with cholesterol or ergosterol does not disprove a link between inhibition of sterol synthesis and algicidal properties. Although it appears likely that the molecular targets of the applied compounds in algae are analogous to those described for higher plants more data are required in order to link the cellular effects definitively to the corresponding enzymic targets.

Synchronized cultures of algae might be a suitable tool for further investigations on sterol or GA biosynthesis and the mode of action of the inhibitors. A correlation of cell development and susceptibility to specific inhibitors of sterol and GA biosynthesis may allow differentiation between different and/or successive biosynthetic steps and their regulation. In *C. fusca* the physiological processes affected by P450 inhibitors and fenpropimorph are triggered for only the last two hours (t_{12}–t_{14}) within the period of cell volume growth (Fig. 1(a)). On the other hand, results obtained with *C. sorokiniana* (Fig. 1(b)) indicate that the target sites of P450 inhibitors and fenpropimorph must differ. Processes susceptible to fenpropimorph in this alga take place within the first two hours after the light/dark change (t_{10}–t_{12}), three to four hours later than those sensitive to propiconazole or prochloraz.

Concerning the algal response to P450 inhibitors, the results obtained fit well with those of a recent study where the *in-vivo* ECOD and EROD activities of *C. sorokiniana* and *C. fusca* have been shown to vary characteristically during the cell cycle. It was suggested that this indicates the appearance of competing endogenous substrate(s) after t_5 and t_{10}, respectively.[14] Although algae, in contrast to higher plants, have not yet been shown to contain substrate-specific different P450 isoforms, it appears likely that P450s, usually involved in physiological processes, can also be involved in the biotransformation of xenobiotics.[38,39]

Thus the strong inhibition of the *in-vivo* *O*-dealkylase activities revealed in algae by pesticides known to be P450 inhibitors is not at all surprising, but has been validated for a reasonable number of compounds. The contrasting data of ABT, an agent which does not inhibit cell division but strongly inhibits its ECOD and EROD activities, might be an indication for distinct P450s involved. In higher plants, ABT has been proposed as a selective inhibitor of cinnamic acid 4-hydroxylase, a P450 isoenzyme which also catalyzes the *O*-dealkylation of 7-ethoxycoumarin and 7-ethoxyresorufin.[22,40] Although there are distinct differences in the sensitivities of both algae to individual compounds, a simplified ranking of the toxicity data indicates some selectivity in the inhibitory properties. Types of inhibition may be arranged in the following order: cell division > phase-I *O*-dealkylation > cell volume growth. However, it is difficult to separate effects on cell division from those on phase-I biotransformation capacity. Although both dealkylases, EROD and ECOD, may not be of physiological importance in algae with respect to cell volume growth and cell division, the cytochrome P450-dependent phase-I biotransformation capacity is established as an important determinant of resistance to pesticides and other xenibiotics. Thus, effects on this detoxification mechanism cannot be ignored as only a contributory factor; such 'side effects' caused by cytochrome P450-interacting plant growth retardants and fungicides have to be considered seriously with regard to residual levels when pesticide mixtures with such compounds are used or successive applications made.

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REFERENCES

1. Durst, F. & Benveniste, I., Cytochrome P450 in plants. In *Cytochrome P450*, ed J. B. Schenkman & H. Greim. Springer-Verlag, Berlin–New York, 1993, pp. 293–309.
2. Cole, D. J., Detoxification and activation of agrochemicals in plants. *Pestic. Sci.*, **42** (1994) 209–22.
3. Grossman, K., Plant growth retardants: their mode of action and benefit for physiological research. In *Progress in Plant Growth Regulation*, ed. C. N. Karssen, L. C. Van Loon & D. Vreugdenhil. Kluwer Academic Publishers, Dordrecht, 1991, pp. 789–97.
4. McFadden, J. J., Gronwald, J. W. & Eberlein C. V., *In-vitro* hydroxylation of bentazon by microsomes from naphthalic anhydride-treated corn shoots. *Biochem. Biophys. Res. Commun.*, **168** (1990) 206–13.

5. Salaün, J.-P., Weissbart, D., Durst, F., Pflieger, P. & Mioskowski, C., Epoxidation of *cis* and *trans* Δ^9-unsaturated lauric acids by a cytochrome P-450-dependent system from higher plant microsomes. *FEBS Lett.*, **246** (1989) 120–26.
6. Fonné-Pfister, R., Simon, A., Salaün, J.-P. & Durst, F. Xenobiotic metabolism in higher plants: involvement of microsomal cytochrome P-450 in aminopyrine *N*-demethylation. *Plant Sci.*, **55** (1988) 9–20.
7. Werck-Reichhart, D., Gabriac, B., Teutsch, H. & Durst, F., Two cytochrome P-450 isoforms catalysing *O*-de-ethylation of ethoxycoumarin and ethoxyresorufin in higher plants. *Biochem. J.*, **270** (1990) 729–35.
8. Rademacher, W., Fritsch, H, Graebe, J. E., Sauter, H. & Jung, J., Tetcyclacis and triazole-type plant growth retardants: their influence on the biosynthesis of gibberellins and other metabolic processes. *Pestic. Sci.*, **21** (1987) 241–52.
9. Taton M., Ullmann, P., Benveniste, P. & Rahier, A., Interaction of triazole fungicides and plant growth regulators with microsomal cytochrome P-450-dependent obtusifoliol-14α-methyl demethylase. *Pestic. Biochem. Physiol.*, **30** (1988) 178–89.
10. Ortiz de Montellano, P. R. & Mathews, J. M., Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. *Biochem. J.*, **195** (1981) 761–4.
11. Wilkinson, C. F., Murray, M. & Marcus C. B., Interactions of methylenedioxyphenyl compounds with cytochrome P-450 and effects on microsomal oxidation. In *Reviews in Biochemical Toxicology 6*, ed. E. Hodgson, J. R. Bend & R. M. Philpot. Elsevier Science Publishers, New York, 1984, pp. 27–63.
12. Cole, D. J. & Owen, W. J., Influence of monooxygenase inhibitors on the metabolism of the herbicide chlorotoluron and metolachlor in cell suspension cultures. *Plant Sci.*, **50** (1987) 13–20.
13. Canivenc, M.-C., Cagnac, B., Cabanne, F. & Scalla R., Induced changes of chlorotoluron metabolism in wheat cell suspension cultures. *Plant Physiol. Biochem.*, **27** (1989) 193–201.
14. Thies, F. & Grimme L. H., *O*-dealkylation of coumarin- and resorufin-ethers by unicellular green algae—kinetic properties of *Chlorella fusca* and *Chlorella sorokiniana*. *Arch. Microbiol.*, **164** (1995) 203–11.
15. Burdon, R. S., Carter, G. A., Clark, T., Cooke, D. T. Croker, S. J., Deas, A. H. B., Hedden, P., James, C. S. & Lenton J. R., Comparative activity of the enantiomers of triadimenol and paclobutrazol as inhibitors of fungal growth and plant sterol and gibberellin biosynthesis. *Pestic. Sci.*, **21** (1987) 253–67.
16. Yates, P. J., Lenton, J. R. & Goad, L. J., Effects of triazole inhibitors of sterol biosynthesis on the free and esterified sterol composition of celery cell suspensions cultures. *Pestic. Sci.*, **39** (1993) 257–65.
17. Cerdon, C., Rahier, A., Taton, M. & Sauvaire, Y., Effects of tetcyclacis on growth and on sterol and sapogenin content in fenugreek. *J. Plant Growth Regul.*, **14** (1995) 15–22.
18. Buchenauer, H. & Röhner, E., Effect of triadimefon and triadimenol on growth of various plant species as well as on gibberellin content and sterol metabolism in shoots of barley seedlings. *Pestic. Biochem. Physiol.*, **15** (1981) 58–70.
19. Kapteyn, J. C., Milling, R. J., Simpson, D. J. & De Waard, M. A., Inhibition of sterol biosynthesis in cell-free extracts of *Botrytis cinerea* by prochloraz and prochloraz analogues. *Pestic Sci.*, **40** (1994) 313–19.
20. Rahier, A. & Taton, M., The 14α-demethylation of obtusifoliol by a cytochrome P-450 monooxygenase from higher plants microsomes. *Biochem. Biophys. Res. Commun.*, **140** (1986) 1064–72.
21. Bollenbacher, W. E., Smith, S. L., Wielgus, J. J. & Gilbert L. I., Evidence for an α-ecdysone cytochrome P-450 mixed function oxidase in insect fat body mitochondria. *Nature (London)* **268** (1977) 660–3.
22. Reichhart, D., Simon, A., Durst, F., Mathews, J. M. & Ortiz de Montellano, P. R., Autocatalytic inactivation of plant cytochrome P-450 enzymes: selective inactivation of cinnamic acid 4-hydroxylase from *Helianthus tuberosus* by 1-aminobenzotriazole. *Arch. Biochem. Biophys.*, **216** (1982) 522–9.
23. Taton, M., Benveniste, P. & Rahier, A., Mechanism of inhibition of sterol biosynthesis enzymes by *N*-substituted morpholines. *Pestic Sci.*, **21** (1987) 269–80.
24. Stearns, M. E. & Brown, D. L., Microtubule organizing centers (MTOCs) of the alga *Polytomella* exert spatial control over microtubule initiation *in vivo* and *in vitro*. *J. Ultrastruct. Res.*, **77** (1981) 366–78.
25. Griggs, D. L., Hedden, P., Temple-Smith, K. E. & Rademacher, W., Inhibition of gibberellin 2β-hydroxylases by acylcyclohexanedione derivatives. *Phytochemistry*, **30** (1991) 2513–17.
26. Rademacher, W., Biochemical effects of plant growth retardants. In *Plant Biochemical Regulators*, ed. H. W. Gausman. Marcell Dekker, Inc., New York, 1991, pp. 169–98.
27. Worthing C. R. & Hance R. J., *The Pesticide Manual*, 9th Edition. British Crop Protection Council, Surrey, 1991, pp. 149–929.
28. Klotz, A. V., Stegeman, J. J. & Walsh, C., An alternative 7-ethoxyresorufin *O*-deethylase activity assay: a continuous visible spectrophometric method for measurement of cytochrome P-450 monooxygenase activity. *Anal. Biochem.*, **140** (1984) 138–45.
29. Grimme, L. H. & Boardman, N. K., Photochemical activities of a particle fraction P_1 obtained from the green alga *Chlorella fusca*. *Biochem. Biophys. Res. Commun.*, **49** (1972) 1617–23.
30. Altenburger, R., Bödeker, W., Faust, M. & Grimme, L. H., Evaluation of the isobologram method for the assessment of mixtures of chemicals. *Ecotoxicol. Environ. Saf.*, **20** (1990) 98–114.
31. Faust, M., Altenburger, R., Boedeker, W. & Grimme, L. H. Algentoxizitätstests mit synchronisierten Kulturen. In *Biologische Testverfahren (Schriftenreihe WaBoLu, Vol. 89)*, ed. K. G. Steinhäuser & P. D. Hansen. G. Fischer, Stuttgart, New York, 1992, pp. 311–22.
32. Boedeker, W., Altenburger, R., Faust, M. & Grimme, L. H., Biometrische Verfahren zur Auswertung von Biotests. In *Biologische Testverfahren (Schriftenreihe WaBoLu, Vol. 89)*, ed. K. G. Steinhäuser & P. D. Hansen. G. Fischer, Stuttgart, New York, 1992, pp. 67–81.
33. Thies, F. & Grimme, L. H., *In-vivo O*-dealkylation of resorufin and coumarin ethers by the green alga *Chlorella fusca* analysed by a rapid and sensitive high-performance liquid chromatographic assay. *J. Chromatogr. (A)*, **684** (1994) 168–73.
34. Großmann, K., Plant growth retardants as tools in physiological research. *Physiol. Plant.*, **78** (1990) 640–8.
35. Bradley, P. M., Plant hormones do have a role in controlling growth and development of algae. *J. Phycol.*, **27** (1991) 317–21.
36. Evans, L. V. & Trewavas, A. J., Is algal development controlled by plant growth substances? *J. Phycol.*, **27** (1991) 322–6.
37. Rezanka, T., Analysis of sterol esters from alga and yeast

by high performance liquid chromatography-mass spectrometry with chemical ionization. *J. Chromatogr.*, **598** (1992) 219–26.
38. Zimmerlein, A. & Durst, F., Arylhydroxylation of the herbicide diclofop by a wheat cytochrome P450 monooxygenase. Substrate specificity and physiological activity. *Plant Physiol.*, **100** (1992) 874–81.
39. Pierrel, M. A., Batard, Y., Kazmaier, M., Mignotte-Vieux, C., Durst, F. & Werck-Reichhart, D., Catalytic properties of the plant cytochrome P450 CYT73 expressed in yeast: substrate specificity of a cinnamate hydroxylase. *Eur. J. Biochem.*, **224** (1994) 835–44.
40. Werck-Reichhart, D., Gabriac, B., Teutsch, H. & Durst, F., Two cytochrome P-450 isoforms catalysing *O*-de-ethylation of ethoxycoumarin and ethoxyresorufin in higher plants. *Biochem. J.*, **270** (1990) 729–35.